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7,12-Dimethylbenz(a)anthracene-Deoxyribonucleoside Adduct Formation 'in vito' Evidence for the Formation and Binding of a Monohydroxymethyl-DMBA (Dimethylbenz(a)anthracene) Metabolite to Rat Liver DNA (Deoxyribonucleic Acid)

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16. ABSTRACT

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7,12-Dimethylbenz[a]anthracene-deoxyribonucleoside adduct formation *in vivo*: evidence for the formation and binding of a monohydroxymethyl-DMBA metabolite to rat liver DNA

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Abstract

The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA) is a potent carcinogen to the female Sprague-Dawley rat, and when administered under conditions that have been shown to produce cancer, results in extensive formation of hydrocarbon-deoxyribonucleoside adducts. Sephadex LH-20 and reverse-phase h.p.l.c. and spectrofluorometric analysis of these adducts demonstrate that at least one adduct results from the binding of 7,12-dimethylbenz[a]anthracene-1,2,3,4-tetrahydro-3,4-dihydroxy-1,2-oxide. In these experiments, employing i.p. administration of the hydrocarbon, a second more polar adduct was observed. Evidence is presented that this adduct results from the formation of a monohydroxymethyl-methylbenz[a]anthracene-A-ring-diol-epoxide. While both of the monohydroxymethyl-DMBA metabolites have been shown to bind cellular DNA following their administration this is the first evidence of monohydroxymethyl-DMBA-deoxyribonucleoside adducts being formed after the administration of DMBA *per se*. The evidence suggests that this more polar adduct is a 7-hydroxymethyl-12-methylbenz[a]anthracene-deoxyribonucleoside adduct.

Introduction

The polycyclic aromatic hydrocarbon 7,12-dimethylbenz-[a]anthracene (DMBA)* (Figure 1, **1**) is a potent carcinogen toward both the rat (1,2) and mouse (3,4). It has been proposed that this compound binds to cellular DNA via the formation of a reactive diol-epoxide in the A-ring (Figure 1, **4**) (5–10) in accordance with "bay region" theory advanced by Jerina and Daly (11).

DMBA is readily metabolized by cellular enzymes in cultured cells (10,12,13) and in cell free systems (10,14,15) to a variety of metabolites involving oxidation at both ring carbons and the exocyclic methyl functions. The two possible monohydroxymethyl derivatives of DMBA, 7-hydroxymethyl-12-methylbenz[a]anthracene (7OHM-12MBA, Figure 1, **2**) and 7-methyl-12-hydroxymethylbenz[a]anthracene (7M-12OHMBA, Figure 1, **3**), are commonly observed in studies of DMBA metabolism (12, 15). Both of these metabolites are weaker carcinogens than DMBA itself (16–20) and bind to cellular DNA at lower levels than the parent hydrocarbon (21,22).

In contrast to previous observations in both mouse embryo cells (21) and mouse skin *in vivo* (22) we now report that some of the hydrocarbon-deoxyribonucleoside adducts formed in rat liver *in vivo* following i.p. administration of DMBA result from hydroxylation on one of the exocyclic methyl groups in addition to formation of an A-ring diol-epoxide. The evidence presented here indicates that this new product is a 7OHM-12MBA-deoxyribonucleoside adduct. This is the first report of 7OHM-12MBA adduct formation following administration of the parent hydrocarbon, DMBA, *per se*.

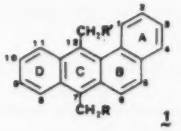
Materials and Methods

Hydrocarbons

DMBA was purchased from Eastman Chemical Co., NY, and was purified within several days of usage by chromatography on silica gel thin layer plate (Eastman, NY, 13179) in cyclohexane:benzene (80:20, v/v). Generally [^{3}H]DMBA (Amersham Corp., IL) and 7,12-[^{14}C-dimethyl]-benz[a]anthracene (New England Nuclear, MA) were mixed with the cold material prior to t.l.c. The specific radioactivities of the [^{3}H] and [^{14}C]DMBA used in this study were 63.8 and 23.5 mCi/mmol, respectively. The samples of cold [^{3}H]7OHM-12MBA were kindly supplied by Dr. David Longfellow of the National Cancer Institute, MD. The final specific radioactivity of this compound was 15.0 mCi/mmol. All preparations were checked by analytical h.p.l.c. before usage and were >96% radiochemically and chemically pure.

Preparation of hydrocarbon-DNA adducts

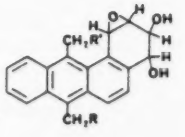
Forty day old Sprague-Dawley (S.D.) female rats were purchased from



1 DMBA R=H R'=H

2 7OHM-12MBA R=OH R'=H

3 7M-12OHMBA R=H R'=OH



4 DMBA-3,4-DIOL-1,2-EPOXIDE R=H R'=H

5 7OHM-12MBA-3,4-DIOL-1,2-EPOXIDE R=OH R'=H

6 7M-12OHMBA-3,4-DIOL-1,2-EPOXIDE R=H R'=OH

Fig. 1. Line structures showing ring numbering and lettering designations for the hydrocarbons and the structures for the bay region diol-epoxides referred to in the text. (See text for abbreviations.)

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***Abbreviations:** DMBA, 7,12-dimethylbenz[a]anthracene; DMBA-diol-epoxide, 7,12-dimethylbenz[a]anthracene-1,2,3,4-tetrahydro-3,4-dihydroxy-1,2-oxide; DMBA-3,4-DHD, 7,12-dimethylbenz[a]anthracene-3,4-dihydrodiol; -DHD, dihydrodiol; h.p.l.c., high pressure liquid chromatography; PNP, 4-(p-nitrobenzyl)-pyridine; 7OHM-12MBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; 7M-12OHMBA, 7-methyl-12-hydroxymethylbenz[a]anthracene; 7OHM-12MBA-diol-epoxide, 7-hydroxymethyl-12-methylbenz[a]anthracene-1,2,3,4-tetrahydro-3,4-dihydroxy-1,2-oxide; S.D., Sprague-Dawley.

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Charles River, MA. These animals were injected i.p. at 50 days of age with 20 μmol of the appropriate hydrocarbon dissolved in either 0.25 ml sesame oil or 0.20 ml dimethylsulfoxide. Pilot studies revealed that the liver DNA binding levels are not influenced by the choice of vehicle.

All animals were housed in temperature controlled rooms (22 – 23°C) with artificial lighting (12 h light), with food and water *ad libitum*. Animals were killed by decapitation and the livers were removed, rinsed in 0.1 M Tris-0.01 M EDTA, weighed and immediately frozen in liquid nitrogen and stored at – 70°C until DNA isolation. All sacrificing and tissue processing were carried out under yellow (Westinghouse FG040) lamps.

DNA isolation

Frozen livers were homogenized in two volumes 0.1 M Tris – 0.1 M EDTA buffer (pH 7.7, 25°C) containing autodigested pronase B (Cal Biochem.) using a Tekmar Tissumizer equipped with large probe at 100 r.p.m. for 15 sec. Homogenates were immediately made 1.5% in SDS and incubated at 37°C for 60 min, following incubation the homogenates were made 6% in 4-aminosalicylate and 1% in NaCl and extracted with one volume chloroform:isoamyl alcohol (24:1, v/v). The resulting aqueous layer was extracted with one volume phenol reagent and after centrifugation (10 000 x *g*, 5°C, 30 min) the nucleic acids were precipitated from the aqueous layer by addition of 1.5 volumes of 2-ethoxyethanol.

The nucleic acid precipitate was washed twice with ethanol and twice with ethyl ether and redissolved in a minimum volume of 0.1 M Tris – 0.1 M EDTA buffer and treated with 50 μg/ml RNase A (heat treated), 50 μg/ml α-amylase for 30 min at 37°C. The solution was made 1% with respect to NaCl and the DNA precipitated by addition of 1.5 volumes of 2-ethoxyethanol. The pellet was washed twice with ethanol and ethyl ether, redissolved in 0.1 M Tris (pH 7.7) and analyzed by reaction of a small aliquot with 3,5-diaminobenzoic acid. These DNA preparations contained < 1% (by weight) protein (BioRad protein assay). The binding level was calculated by digesting an aliquot of the DNA in 35% perchloric acid (80°C, 20 min) followed by the addition of PCS II (Amersham) and liquid scintillation analysis.

DNA hydrolysis

The purified DNA was hydrolyzed by the addition of 0.3 M Tris buffer with 0.01 M $MgCl_2$, 0.005 M $CaCl_2$, 0.001 M $ZnCl_2$ (pH 8.3 at 25°C) and small amounts of DNase I, and snake venom phosphodiesterase (Worthington, NJ). After incubation at 37°C for 4 – 6 h bacterial alkaline phosphatase (Worthington, NJ) was added and the solution incubated at 37°C for 14 – 16 h in the dark. Samples were checked for complete hydrolysis to the nucleoside level by co-chromatography with deoxyribonucleoside and deoxyribonucleotide standards on cellulose t.l.c. (Eastman Kodak 13254) developed in a solvent of 1 M ammonium acetate:ethanol, (33:67, v/v) pH 7.5.

Fluorescence spectroscopy

Fluorescence spectra were recorded on an AMINCO SPF 500 instrument equipped with a lamp ratio accessory. Spectra were taken in h.p.l.c. grade methanol at room temperature in quartz cells and are given uncorrected.

Chromatography

The Sephadex LH-20 system, previously described in detail (10), consisted of a 0.9 x 33 cm column (5.7 gm gel) eluted with a 0 – 100% methanol in water gradient. One hundred 2 ml fractions were collected for liquid scintillation counting. A small amount of 4-(p-nitrobenzyl)-pyridine (PNP) was added to each hydrolysate immediately before chromatography to serve as an u.v. reference marker.

The hydrocarbon-deoxyribonucleoside adducts were separated from the unmodified deoxyribonucleosides in the hydrolysates by two extractions with an equal volume of water-saturated n-butanol. The n-butanol phase was dried under N_2 and redissolved in 35% methanol in water for h.p.l.c. The h.p.l.c. gradient system, using a DuPont Zorbax ODS column (4.6 x 250 mm), consisted of a linear 45 min, 35 – 80% methanol in water gradient eluted at 1 ml/min by an LDC chromatographic system controlled by an LDC-Chromatographic Control Module (CCM). The effluent was monitored by an LDC spectromonitor-III u.v. detector set at 290 nm and an LDC fluoromonitor-III with excitation at 254 nm and a 470 nm emission filter. Fractions of 0.5 ml were collected using an ISCO model 1800 fraction collector fitted with a model 590 flow interruptor. The ninety fractions were counted in an LKB model 1216 scintillation counter equipped with a DPM-PLOT package, after mixing with 5 ml PCS II (Amersham).

The system for preparing DMBA-deoxyribonucleoside adducts from Syrian hamster embryo cells has been previously described (10).

Results

DNA isolated 48 h after i.p. administration of 20 μmol [^{3}H]DMBA, from the livers of 50 day old female S.D. rats contains 4.6 ± 1.9 (N = 4) μmol DMBA/mol deoxyribonucleotide ± standard deviation. This is a somewhat lower binding level than observed previously in the liver following i.v. administration (23).

When hydrolysates of liver DNA from rats previously treated with [^{3}H]DMBA were chromatographed on Sephadex LH-20 the profile shown in Figure 2 was obtained. At least four peaks of radioactivity are invariably observed in these chromatograms. Radioactivity eluting in the polar (high water) portion of the gradient (LH1 and LH2) has consistently been observed by other investigators (24,25) studying DMBA – DNA binding. The chemical nature of this early polar material has not been characterized and varies with experiment as a percentage of the total radioactivity isolated with the DNA. Two peaks of radioactivity are routinely observed in the mid-regions of these LH-20 chromatograms: LH3, fractions 48 – 53, and LH4, fractions 60 – 67. LH4 is chromatographically identical to that observed in DNA hydrolysates obtained when Syrian hamster embryo cells, previously treated with [^{3}H]DMBA, were analyzed on this system (Figure 2) (10). In contrast the other peak, LH3 which elutes in this system with the u.v. standard, PNP, is not found in digests from cultured rodent cells either in our laboratory (10) or those of other investigators (5,9,26,27). When DMBA is administered by the i.p. route the adducts LH3 and LH4 are found in essentially equal amounts in the liver at 48 h (Figure 2).

In order to further resolve these adducts the DNA hydrolysates were extracted with water saturated n-butanol and the organic phase analyzed on Zorbax ODS columns. Figure 3 depicts the h.p.l.c. of the n-butanol phase resulting from the extraction of the DNA hydrolysate from livers of four animals treated 48 h previously with [^{3}H]DMBA. Subsequent experiments confirmed that peak III (h.p.l.c.) corresponds to LH3 (Sephadex LH-20) and peak IV (h.p.l.c.) and the small satellite peaks correspond to LH4 (Sephadex LH-20). These results are summarized in the h.p.l.c. profile of the n-butanol extract shown in Figure 3. Peaks I and II (h.p.l.c.) clearly correspond to LH1 and LH2 (Sephadex LH-20) and can be separated on ODS columns by employing gradients starting with higher percentages of water. In these experiments ~ 25 – 50% of the radioactivity in the DNA hydrolysate is extractable by n-butanol. That which remains in the aqueous phase chromatographs as I and II, exclusively.

Quantities of III and IV sufficient for spectrofluorometric analysis were prepared from livers of five adult (400 g) S.D. female rats dosed i.p. 48 h prior to sacrifice with 0.2 ml of DMSO containing 80 μmol of [^{3}H]DMBA. The pooled hydrolysates were partially purified on Sephadex LH-20 and subjected to further purification by h.p.l.c. (both III and IV are conveniently monitored by an h.p.l.c. fluorescence detector). The fluorescence spectra of III and IV, in methanol, are shown in Figure 4. The emission spectrum of IV exhibits two broad bands at ~ 430 nm and 450 nm while that of III is very similar but shifted by 5 – 7 nm to the shorter wavelengths. These spectra are very similar to those reported earlier for A-ring DMBA-deoxyribonucleoside adducts (5,7,9).

Figure 5 depicts the dual-isotope h.p.l.c. profile obtained from the co-chromatography of n-butanol extracts of liver DNA hydrolysates of two 50 day old female S.D. rats treated i.p. 48 h prior to sacrifice, one with 20 μmol of [^{14}C]DMBA and the other with 20 μmol of [^{3}H]7OHM-12MBA. The [^{3}H]7OHM-12MBA – deoxyribonucleoside adduct clearly

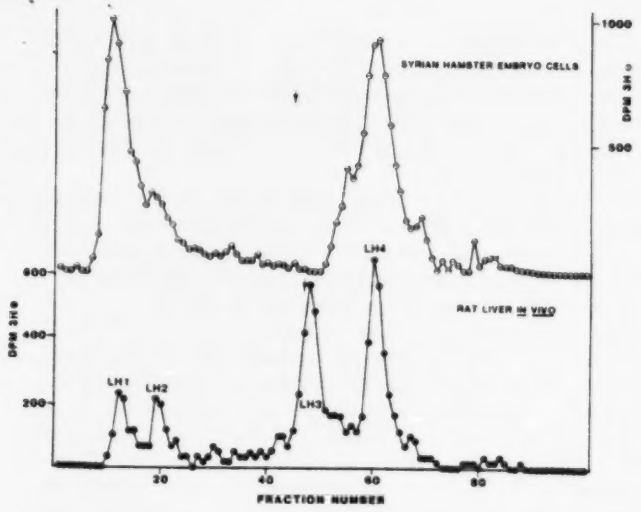

Fig. 2. Upper: Sephadex LH-20 chromatogram of a DNA hydrolysate from Syrian hamster embryo cells, treated 24 h previously with [^{3}H]DMBA. Lower: Sephadex LH-20 chromatogram of the n-butanol extract of a liver DNA hydrolysate from female S.D. rats previously treated i.p. with [^{3}H]DMBA. Arrows indicate the elution of the u.v. marker 4-(p-nitrobenzyl)-pyridine.

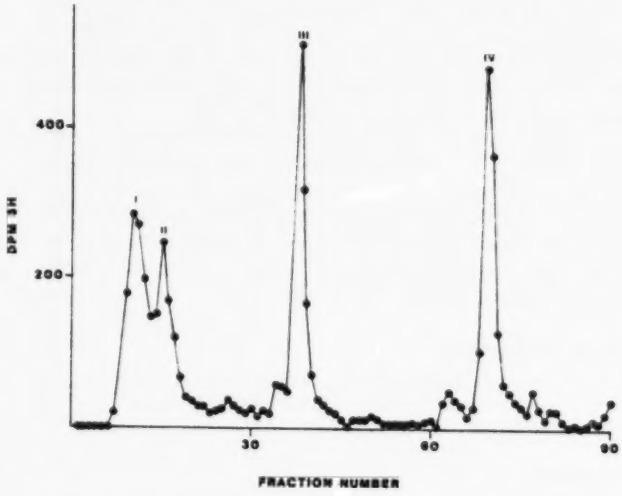

Fig. 3. H.p.l.c. chromatogram of the n-butanol extract of a liver DNA hydrolysate from female S.D. rats treated i.p. 48 h previously with [^{3}H]DMBA.

chromatographs with III from the [^{14}C]DMBA-deoxyribonucleoside adducts.

Discussion

Jerina and Daly have proposed a generalized theory for activation of angular polycyclic aromatic hydrocarbons involving formation of a vicinal diol-epoxide in the "bay region" which subsequently produces a reactive benzylic carbonium ion. Such electrophilic species can bond covalently to cellular macromolecules including DNA and it has been proposed that the formation of such carcinogen DNA adducts are initiation events in the processes of chemical carcinogenesis (28).

DMBA (Figure 1, **1**) is the most potent PAH carcinogen yet studied toward the mammary gland of S.D. female rats

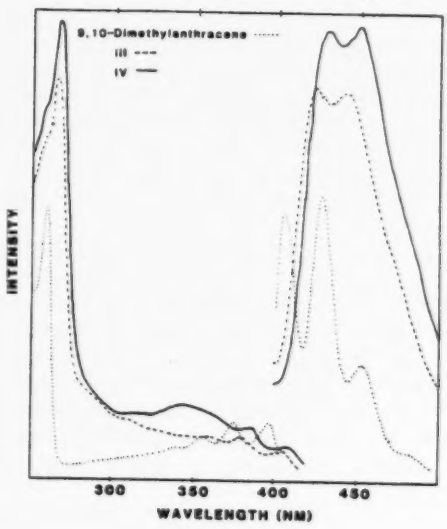

Fig. 4. Fluorescence spectra of 9,10-dimethylanthracene (...), adduct III (h.p.l.c.) (—) and adduct IV (h.p.l.c.) (__). Uncorrected spectra taken in methanol at room temperature. Excitation spectra recorded with emission at 440 nm, emission with excitation at 267 nm.

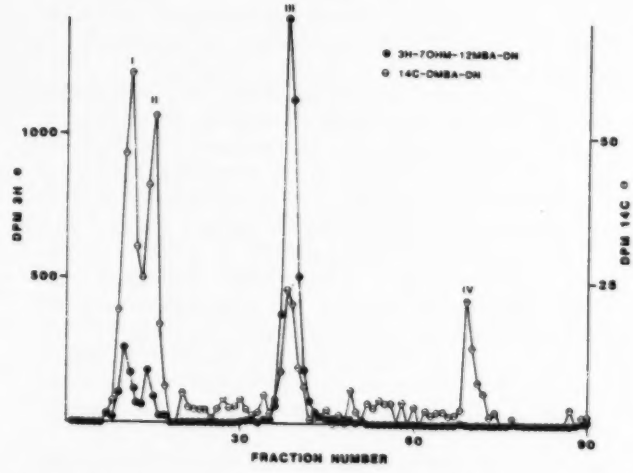

Fig. 5. H.p.l.c. chromatogram of the n-butanol extract of a mixture of liver DNA hydrolysates from female S.D. rats treated i.p. 48 h previously with [^{3}H]7OHM-12MBA or [^{14}C]DMBA (see **Results** section).

and as little as 20 μmol given either i.v. (29) or i.p. (1) will produce a high yield of mammary adenocarcinomas in 5–6 weeks. The bay region for DMBA is the area between the C1 and C12 positions and the expected critical DNA binding metabolite of DMBA is predicted to be a diol-epoxide in the A-ring (*ie.* C1, C2, C3 and C4, Figure 1, **4**). While this metabolite has never been chemically synthesized a great deal of indirect evidence indicates it is the metabolite responsible for at least some of the hydrocarbon-DNA binding observed in whole cells treated with DMBA. The major DMBA-deoxyribonucleoside adduct observed on Sephadex LH-20 chromatograms of DNA digests from cultured Syrian hamster embryo cells is photosensitive (26,27), contains a diol group (9) and has a fluorescence spectrum consistent with a DMBA moiety saturated in the A-ring (7). Likewise the major DMBA-deoxyribonucleoside adducts obtained from

cultured mouse embryo cells (5,6), mouse skin *in vivo* (8) and the reaction of DMBA-3,4-DHD, 3-chloroperbenzoic acid and calf thymus DNA (8) also have similar chromatographic and spectrofluorometric properties. Further, the precursor to the diol-epoxide, DMBA-3,4-DHD, has been shown to be more carcinogenic than the parent hydrocarbon in the newborn mouse (17) and a more potent initiator than DMBA in mouse skin (18,30).

In these studies on S.D. female rat liver one of the major DMBA-deoxyribonucleoside adducts from Sephadex LH-20 columns, LH4, co-chromatographs with the major adduct from Syrian hamster embryo cells (Figure 2). Further, when LH4 is subject to further purification on reverse-phase h.p.l.c. columns the major component IV has a fluorescence spectrum in excellent agreement with that reported by Moschel *et al.* (5,6) and others (7,8) for DMBA-deoxyribonucleoside adducts containing a hydrocarbon with a saturated A-ring.

In addition to LH4, however, these Sephadex LH-20 chromatograms from rat liver DNA hydrolysates also contain another major component, LH3, not found in hamster embryo DNA digests either in our laboratory (Figure 2) (10) or others (5,9,26). On our Sephadex LH-20 system this product, LH3, elutes approximately with PNP the u.v. marker. On the Sephadex LH-20 system of Baird and Brookes (31) it elutes ~12 ml before PNP or where 7OHM-12MBA-deoxyribonucleoside adducts have been shown to elute (21).

When LH3 is further purified on Zorbax-ODS >85–90% of the activity elutes as a single component, III (Figure 3). When sufficient quantities of III for fluorescence studies were prepared, the spectrum (Figure 4) was in excellent agreement with that reported by Moschel *et al.* (6) for 7OHM-12MBA-deoxyribonucleoside adducts. For comparison Figure 4 also shows the spectrum of 9,10-dimethylanthracene, a model fluorochrome for A-ring saturated DMBA. It is noteworthy that the excitation maxima for the three compounds are essentially identical and that the emission spectrum for III is shifted 3–5 nm to shorter wavelengths relative to IV just as observed by Moschel *et al.* (6) for 7OHM-12MBA-deoxyribonucleoside adducts relative to DMBA-deoxyribonucleoside adducts.

Lastly, in a double label experiment, Figure 5 shows that the tritium labeled 7OHM-12MBA-deoxyribonucleoside adduct co-chromatographs exactly, on h.p.l.c., with the [^{14}C] labeled III obtained from rat liver after [^{14}C]DMBA administration.

The possibility that III (or LH3) is a deoxyribonucleoside adduct of the isomeric 7M-12OHMBA (or a mixture of 7OHM-12MBA and 7M-12OHMBA adducts) has not been rigorously eliminated. However, MacNicoll *et al.* (22) have shown that deoxyribonucleoside adducts from these two isomers are readily separated on Sephadex LH-20 and in our hands III (or LH3) co-chromatographs with the 7OHM-12MBA-deoxyribonucleoside adduct on both Sephadex LH-20 (data not shown) and h.p.l.c. (Figure 5). When LH3 is re-chromatographed on h.p.l.c. a small (~10%) lead-in peak of radioactivity before III is invariably observed (Figure 3). The identity of this material (not sufficient for spectrofluorometric analysis) is unknown. It has also been shown by Yang *et al.* that hydroxylation of the C12 methyl reduces both microsomal induced metabolism (32) and DNA binding (33) relative to C7 methyl hydroxylation. Likewise in mouse skin 7M-12OHMBA binds to DNA only about one third as extensively as the isomeric 7OHM-12MBA (22). Finally, in studies with rat liver microsomes 7OHM-12MBA is a major metabolite of DMBA oxidation while 7M-12OHMBA is a relatively minor one (14,15) and in this system the 7M-12OHMBA is a very poor substrate for oxidation at the 3,4-bond relative to 7OHM-12MBA (32).

The significance of these observations to the carcinogenicity of DMBA are not certain. In the mouse skin tumor initiation-promotion assay (18,19), the newborn mouse (17) and the rat mammary gland (16), 7OHM-12MBA has been shown to be a weaker carcinogen than DMBA itself. On the other hand, the 3,4-DHD's of both DMBA and 7OHM-12MBA were found to be more potent than DMBA itself (17,18). In the case of the other monohydroxymethyl derivative, 7M-12OHMBA, both it and its 3,4-DHD tend to be considerably less active than DMBA (18). The reduced activity of the 7OHM-12MBA relative to DMBA may be related to its reduced ability to cross cell membranes (16). However, in these studies the compound administered is DMBA and presumably the conversion to 7OHM-12MBA and subsequent A-ring activation and DNA-binding occur in the cell. Thus, the critical question is what is the effect of a 7OHM-12MBA-deoxyribonucleoside adduct relative to a DMBA one? This question is being pursued in our laboratory.

It should be noted here that except when the hydrocarbon is administered during liver regeneration (34,35) DMBA is not oncogenic to the female S.D. rat liver. In these studies we used the i.p. route of administration in order to generate sufficient amounts of adduct for spectral studies.

In summary, in the rat *in vivo*, unlike the case of cultured rodent cells (5,10,21) or mouse skin *in vivo* (8,22), DMBA is activated via "bay region" metabolism to form a diol-epoxide in addition to being hydroxylated at one of the exocyclic methyl groups (Figure 1, 5,6). At present the data indicates that this new product is most probably a 7OHM-12MBA-deoxyribonucleoside adduct.

Acknowledgments

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